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Identification and characterization of a novel nasopharyngeal carcinoma-associated peptide: NAP-1

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Abstract

Nasopharyngeal carcinoma (NPC) is one of the most commons cancers in Southeast Asia and Southern China. Several NPC-associated genes have been so far described and here we describe the identification and the characterization of a novel nasopharyngeal carcinoma-associated peptide: NAP-1. NAP-1 was identified with the human genome draft searching method combined with nested PCR mapping of the chromosome 4q13 region. NAP-1 encodes an 85 amino acid alkaline peptide with a calculated isoelectric point of 9.3, three phosphorilation sites and a proline-rich region. Northern blot analysis revealed that NAP-1 is expressed as a 0.6 kb transcript in normal lymph nodes and trachea. In addition, reverse transcription (RT)-PCR showed that NAP-1 is expressed not only in NPC but in normal nasopharynx (NP) and various other tumors and tissues of the head and neck including: tonsils, lymph nodes, carcinoma of the tonsil, T cell lymphomas, squamous cell carcinoma of the hard palate, papilloma of the nasopharynx, nasopharyngitis, lymphoma of the tongue root and follicular dendritic cells (FDC). In addition, NAP-1 is not expressed in normal tissues or tumors from other anatomical regions and was not expressed by NPC cell lines. Surprisingly, differential RT-PCR demonstrated decreased expression of NAP-1 in NPC compared with paired NP biopsies in 42.5 % of cases (17 out of 40). In addition, in situ hybridization and immunohistochemistry demonstrated that NAP-1 is expressed by $S100^+$ $CD35^+$ FDCs of the germinal center and not in other normal immune cells infiltrating NP or NPC. Therefore, it is likely that NAP-1 is secreted by FDC in the NP and may play an immune modulatory role in NPC.

Background

Nasopharyngeal carcinoma (NPC) is an endemic cancer with high incidence in Southeast Asia and Southern China with obvious inclination toward racial and geographic influence. Epidemiological studies indicate that "three hits and multiple steps" may be necessary for the development of NPC. These include genetic predisposition, Epstein – Barr virus (EBV) infection and environmental conditions associated with dietary habits that may lead to chemical carcinogenesis [1]. Among the genetic factors, mutations leading to over expression of oncogenes such as c-myc or H-ras or altered expression of tumor

suppressor genes such as p53 and p16 have been described [2]. However, to these days there is no conclusive molecular understanding of the pathogenesis of NPC and the quest for relevant associations between gene expression patterns in this disease remains relevant.

For this reason we queried available data bases for the identification of putative genetic associations with NPC. By exploring UniGene we could partition GeneBank sequences according to gene-oriented clusters that segregate genes according to their expression in various tissues or diseases. At the same time clusters can be created based on sequence homology at the genomic or transcriptional level. Consequently each UniGene cluster contains sequences most likely to represent a unique gene and information about its tissue distribution. Often expression sequence tags (EST) are identified for which no annotation is available and by assembling available information. It is sometimes possible to perform an electronic prolongation of the relevant EST to construct a putative gene identity. Traditional cDNA library screening can subsequently offer the opportunity to expand knowledge on the identified genes by utilizing information collected from various data bases. Therefore, we utilized this Human Genome Searching Method combined with nested PCR to extend available sequence information on genes of putative interest with particular focus on the characterization of the 5' end and/or the full length of available cDNA libraries [3]. In this fashion, an EST contig was obtained from which a gene associated with NPC was characterized. The gene was named nasopharyngeal carcinoma-associated peptide (NAP)-1 and characterization of its pattern of expression in normal and cancerous tissues was undertaken.

Results

A search was performed on the UniGene data base querying for *Homo. sapiens* nasopharynx. Thirty seven NP-related UniGene clusters were identified. Sixteen of the 37 clusters contained novel not previously annotated sequences. Among those we focused our attention on 4 ESTs (BG222624 mapped chromosome to 5q, BG231276 mapped to 2p, BG231174 mapped to 3p and BG231197 mapped to 4q) located in chromosomal areas in which a high frequency of amplifications or deletions have been reported [4-6]. No differential expression between NPC and paired NP specimens could be observed by RT-PCR for three ESTs (BG222624, BG231276, BG231174) while differences were noted for the one mapped on chromosome 4q.

Therefore, we focused our attention an the latter gene and an EST contig (502 bp) was assembled by combining information from 4 highly homologous ESTs (GenBank accession number CB140082, AI332560, BG059093 and AI701589) according to EST BLAST searches using a cDNA fragment (GenBank accession number BG231197) as electronic probe. Electronic prolongation by EST contig did not reveal a complete open reading frame. Bioinformatics analysis suggested the contig resembled a human peptide precursor secreted by follicular dendritic cells (FDC-SP) (GenBank accession number AF435080) [10] with a 77% homology. No stop codons in frame with the cDNA 5' end start codon of the FDC-SP could be identified. The results suggested that the full-length cDNAs could be obtained by combining the Human Genome Draft Searching Method with nested PCR (Figure 1a). Northern blot analysis revealed that the gene produced a transcript (0.6 kb) that could be identified among normal tissues only in lymph nodes and trachea (Figure 1b).

We named the novel gene nasopharyngeal carcinoma-associated peptide (NAP-1; GenBank Accession number AY190326). The length of NAP-1 cDNA is 573 bp with an ORF from 113 bp to 370 bp. NAP-1 encodes a 9,700 Dalton protein composed of 85 amino acids. The predicted amino acid sequence of the NAP-1 protein is: MKKVLLLITAILAVAVGFPVSQDQEREKRSISDSDELASGF FVFPYPYPFRPLPPIPFPRFPWFRRNFPIPIPESAPT-TPLPSEK. NAP-1 maps in chromosome 4q13 and the genomic sequence spans 9,179 bp that include 5 exons and 4 introns (Table 1). No other genes homologous to NAP-1 could be identified. Bioinformatics analysis indicated that NAP-1 is a alkaline protein with a theoretic isoelectric point of 9.3. The protein is most likely secreted and has 3 phosphorylation sites and 1 proline-rich region.

Differential RT-PCR results suggested that NAP-1 is expressed in NP, NPC, tonsil, lymph node, carcinoma of the tonsil, T-cell lymphoma, squamous cell carcinoma of the hard palate, papilloma of the nasopharynx, chronic nasopharyngitis, lymphoma of the base of the tongue and FDC lines. NAP-1 expression was not found in other normal or tumor biopsies nor in NPC cell lines. (Table 2). These results suggest that NAP-1 gene maybe expressed in immune cells of the NP and in NPC stroma. In addition, differential RT-PCR analysis revealed decreased expression of NAP-1 in 17 of 40 NPC compared with paired NP specimens (42.5%). In 6 cases (15%) NAP-1 expression was higher in NPC compared with NP while there was no difference in expression in the remaining 17 cases (42.5%, Table 3). Possibly, different patterns of expression could be related to the immune status of individual NPC.

In situ hybridization and immunohistochemistry suggested that the NAP-1 peptide is expressed in $S100^+$ $CD35^+$ FDC of the germinal center and not in other stromal immune cells in NPC and NP tissues (Figure 2). RT-PCR, Western Blotting, immunohistochemistry and in situ hybridization of FDC lines further demonstrated that

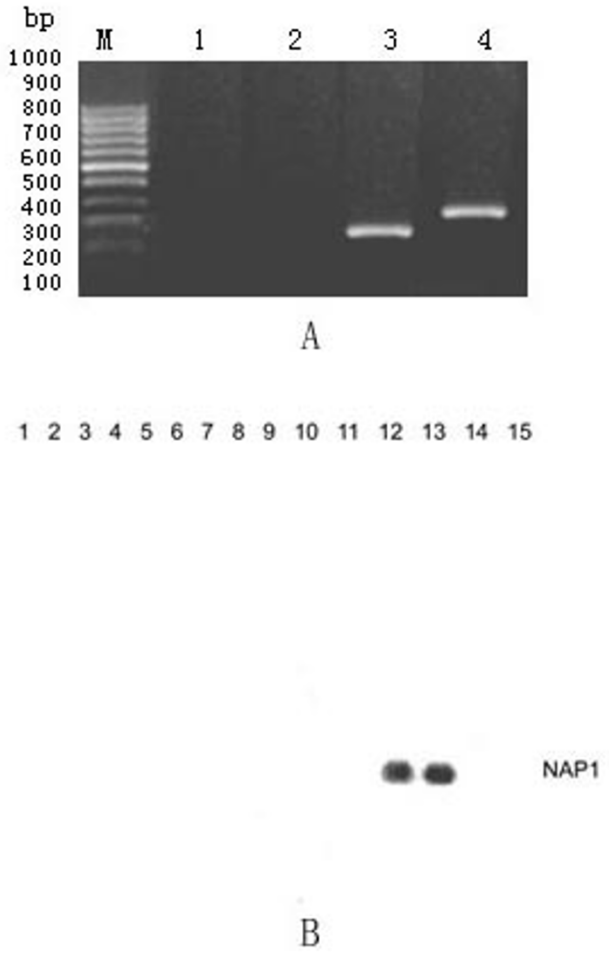

Figure 1
Cloning and identification of NAP-1 gene A: Identification of 5' end's PCR product of the gene by using human genome draft searching methodcombined with nested PCR 1. pFRW-2 and pRVS-3(no); 2. pFRW-2 and pRVS-2(no); 3. pFRW-3 and pRVS-3(176 bp); 4. pFRW-3 and pRVS-2(282 bp) M: DNA ladder **B: Northern blots analysis of the gene in 15 kinds of human tissues** 1. heart; 2. brain; 3. placenta; 4. lung; 5. liver; 6. skeletal muscle; 7. kidney; 8. pancreas; 9. stomach; 10. thyroid; 11. spinal cord; 12. lymph node; 13. trachea; 14. adrenal gland; 15. bone marrow

NAP-1 is a protein specifically secreted by these cells (Figure 3).

Discussion

Several NPC-associated genes have been described in the past. In this study, we describe the identification of a novel NPC-associated peptide that we called NAP-1. NAP-1 was identified searching the human genome data based and confirmed in histologically confirmed NP biopsies using nested PCR. Northern blot analysis revealed that NAP-1 is expressed only in lymph node and trachea among all the normal tissues tested (not in heart, brain, placenta, lung, liver, skeletal muscle, kidney, pancreas, stomach, thyroid, spinal cord, adrenal gland and bone marrow). This result was concordant with RT-PCR analysis that identified NAP-1 expression in normal, inflamed or cancerous nasopharynx biopsies but not in other normal or cancerous biopsies. In addition, since NAP-1 was found to be expressed by FDC but not NPC cell lines it is likely that this peptide is a component of the immune response in NP and may play a modulatory role in the context of NPC. Indeed, common markers for dendritic cells in tumor stroma such as S100 or specific for FDC such CD35 suggest specific expression of NAP-1 by these cells [11-13] as both in situ hybridization and immunohistochemistry pointed to its expression by $S100^+$ $CD35^+$ FDC and not in other stromal immune cells either in NP or NPC tissues (Figure 2). Interestingly, NAP-1 was not observed in the spleen but its expression was noted in the trachea. Since spleen tissue contains mostly primary follicles, we favor the hypothesis that NAP-1 expression is low or absent in primary follicles but is up-regulated in the follicles during germinal center formation by activated FDC, which are present in primary and secondary follicles [14-16]. FDC are present mainly in the germinal center of lymph node paracortex but they can also be found in the germinal center of follicles in mucosa-associated lymphoid tissues such as tonsils and dispersion lymph tissue in other parts of the pharynx. A distinctive characteristic of FDCs is the ability to trap immune complexes and act as antigen depots during the germinal center response. FDCs play a central role in organization of the follicular structure that supports B cell survival, affinity maturation of antibody responses and the generation of long-lived B cell memory [17-19]. Our results suggest that NAP-1 is secreted by FDC and may modulate lymphoid and presumably modulate directly or indirectly NPC cancer cell growth. Thus, expression of NAP-1 may be related to the local immune status of NPC although it's function(s) remains unknown.

Western blotting on FDC lines demonstrated that the NAP-1 protein is secreted by FDC although definitive sequencing of the gene product detected in FDC supernatants was beyond the scope of this study. In addition, we are planning to test whether NAP-1 expression is exclusively restricted to FDC or other antigen presenting cells such as monocyte derived DC activated *in vitro* could also express NAP-1. It is possible that NAP-1 could be a marker of the status of function of monocytes, immature or matured DC [20-22]. This hypothesis should be tested in the future since it may also provide an easier reagent to

Table 1: Exon-intron junctions of the NAP-1 gene

Exon	Exon size(bp)	5'splice donor	Intron size(bp)	3'splice acceptor	Intron
1	112	TTTGAG**gt**aaga	5012		1
2	57	TTCCCA**gt**aagt	1742	ttgc**ag**ATGAAG	2
3	33	AGAAGT**gt**aagt	993	ctttt**ag**GTCTCT	3
4	196	AACCTG**gt**aagt	869	caac**ag**ATCAGT	4
5	165			ttttc**ag**GTCACC	

Uppercase and lowercase letters indicate exon and intron sequences respectively. Conserved splice donor and acceptor dinucleotide sequences are indicated in bold.

Table 2: Expression in other normal, tumor biopsies and cell lines by differential RT-PCR

case number & specimen	IA	case number & specimen	IA
1 tonsil	0.78	2 lymph node	1.00
3 tonsil carcinoma	0.45	4 T- cell lymphoma	0.86
5 tongue root lymphoma	0.35	6 hard palate squamous cell carcinoma	0.39
7 nasopharynx papilloma	0.17	8 chronic nasopharyngitis	0.59
9 follicular dendritic cell line	1.02	10 chronic inflammation of vocal cords	0.00
11 larynx carcinoma	0.00	12 epiglottis carcinoma	0.00
13 hypopharynx carcinoma	0.00	14 cerebra	0.00
15 glioma	0.00	16 ileum	0.00
17 liver, liver cancer	0.00	18 kidney	0.00
19 stomach, stomach cancer	0.00	20 parotid, spleen	0.00
21 fetal heart, uterus	0.00	22 rectum, rectal cancer	0.00
23 CNE-1	0.00	24 CNE-2	0.00
25 HNE-3	0.00	26 5–8F	0.00

study the relationship between immune and NPC cells in different in vitro or in vivo models.

We did not find point mutations and other aberrations in the coding region of the NAP-1 gene by PCR-SSCP (data not shown). In addition, analysis of the NAP-1 gene genomic region did not identified possible CpG island in the 5' non-coding region of NAP-1. Therefore, we believe that decreased expression of NAP-1 in NPC compare with NP represents more a physiopathological fluctuation in the number and function of FDC that populate various NPC biopsies.

Conclusions

In summary, NAP-1 is a protein probably secreted by FDC. NAP-1 might only be expressed in FDC of tonsil, lymph node, tonsil carcinoma, lymphoma, NPC and NP tissue stroma. Its expression is decreased in NPC compared with paired NP biopsies and this differential expression may be associated with local immune status of NPC.

Methods

GeneBank data searching and EST assembly

Gene identifications were on UniGene querying for *Homo. sapiens* nasopharynx. Among genes possibly newly associated with NP we focus on those that resided in chromosomal regions frequency characterized by amplification or deletion in the context of NPC [4-6]. EST contigs where then assembled according to homology ranking according to BLAST searches using a cDNA-based electronic probe.

Tissue samples, cells, cell culture and RNA extract

NPC and paired NP biopsies were obtained at Xiangya Hospital, Central South University, Changsha, China. Other normal and tumor biopsies were also obtained at Xiangya Hospital and included: tonsil, lymph node, carcinoma of the tonsil, carcinoma of the larynx, T-cell lymphoma, carcinoma of the hypo-pharynx, squamous cell carcinoma of the hard palate, papilloma of the nasopharynx, chronic naso-pharyngitis, lymphoma of the base of the tongue, chronically inflamed vocal cords, carcinoma of the epiglottis, cerebral glioma, normal lung, lung cancer, liver, liver cancer, kidney, ileum, stomach, stomach

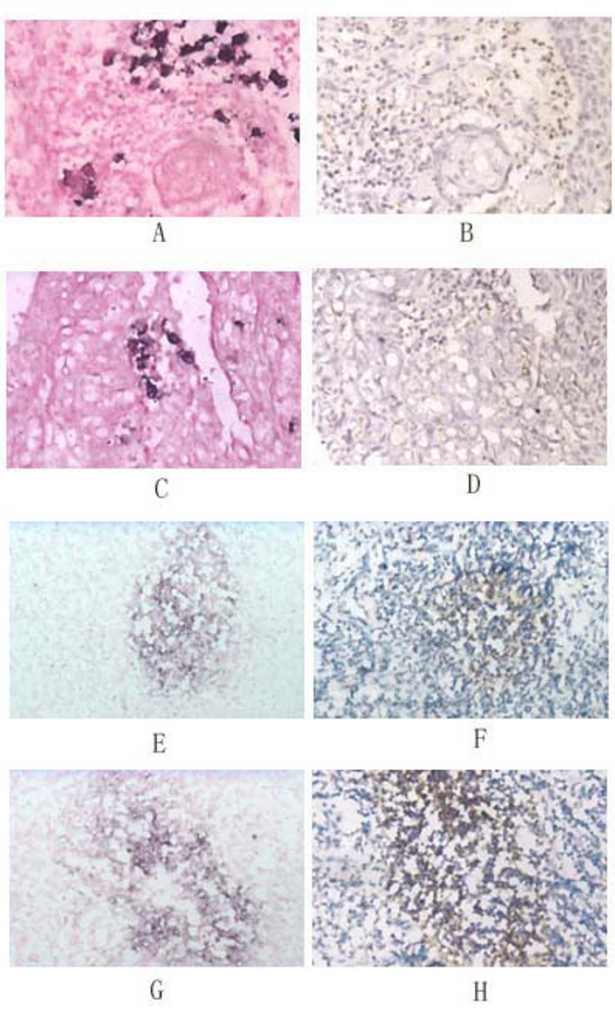

Figure 2
Expression of NAP1 gene in S100$^+$ CD35$^+$ FDC of germinal center not in other stromal immune cells of NP and NPC biopsies. A: In situ hybridization by Dig labelled NAP-1 cDNA probe in NP biopsies (×300); B: Immunohistochemistry by Mouse Anti-S100 Monoclonal antibody in NP biopsies (×300); C: In situ hybridization by Dig labelled NAP-1 cDNA probe in NPC biopsies (×300); D: Immunohistochemistry by Mouse Anti-S100 Monoclonal antibody in NPC biopsies (×300); E: In situ hybridization by Dig labelled NAP-1 cDNA probe in NP biopsies (×300); F: Immunohistochemistry by Mouse Anti-CD35 Monoclonal antibody in NP biopsies (×300); G: In situ hybridization by Dig labelled NAP-1 cDNA probe in NPC biopsies (×300); H: Immunohistochemistry by Mouse Anti-CD35 Monoclonal antibody in NPC biopsies (×300)

cancer, parotid, spleen, fetal heart, uterus, rectum and rectal cancer. The taxonomy of all samples was confirmed by histological examination. The NPC cell lines, CNE-1, CNE-2, HNE-3 and B95-8F were obtained from the Cancer Research Institute, Xiangya School of Medicine, Central South University. NPC cell lines were maintained in RPMI 1640 medium supplemented with 10% FBS, 50,000 units lit.$^{-1}$ Penicillin, streptomycin 50 mg lit.$^{-1}$ at 37°C, in humidified conditions at 5 % CO_2 and 95 % air [4-6]. Follicular dendritic cells lines (HK cells) were kindly provided by Choi YS from the Cancer Research Institute of The First Military Medical University of China. FDC line were cultured by the method as described by Dr. Choi YS [7-9]. Total RNA was extracted from biopsy specimens and cell lines using Trizol reagent (Gibco/BRL Life Technologies, Inc., Gaithersburg, MD, USA).

Differential RT-PCR

Total RNA was digested by RNase-free DnaseI (Roche Diagnostics Corporation, Indianapoils, IN, USA), 37°C, 1 hours. The reaction product was dissolved in double distilled water treated by DEPC. RNA was diluted to 200 μl, wiped off protein with Trizol, and finally dissolved in double distilled water treated by DEPC. For RT-PCR total RNA was reversed transcribed using a commercially available Reverse Transcription System (Promega Corporation, Madison WI, USA). A 20 μl reaction was prepared by adding $MgCl_2$ (25 mM) 4 μl, 10 × Buffer 2 μl, dNTPs(10 mM) 2 μl, RNasin (40U/μl) 0.5 μl, AMV reverse enzyme (24 U/μl) 0.6 μL, Oligo (dT) 1 μl, RNA 2 μg. The reaction was performed at 42°C for 30 minutes followed by heating at 99°C for 5 minutes and incubation at 0–5°C for 5 minutes. The resulting cDNA samples were subjected to PCR amplification using NAP-1's specific primers CAGCGTCAGAGAGAAAGAAC and GTGTGCTATTTCAATTACATTTG (pFRW-1/pRVS-1) or GAPDH control primers AAGCCCATCACCATCTTCCA and CCTGCTTCACCACCTTCTTG. 2 μl 10 × PCR reaction buffer, 0.4 μl Taq DNA polymerase(3U/ μl) (TaKaRa Bio Inc., Otsu, Shiga, Japan), 0.4 μl dNTPs(10 mM), 0.2 μL 20 μM pFRW, 0.2 μl 20 μM pRVS, 0.8 μl biopsies or cells' cDNA were contained in a 20 μl amplification reaction. Amplifications were carried out in a Biometra thermal cycler with the following program: 95°C for 5 minutes, 32 cycles of 94°C for 40 seconds, 58°C for 30 seconds, 72°C for 50 seconds and a final extension at 72°C for 5 minutes. The resulting PCR products were run on agarose gels and stained with ethidium bromide.

Grey-scale scanning and data analysis

The resulting PCR products were scanned by a Gel Scanner (Pharmacia Biotech, Inc., Piscataway, NJ, USA). Scanning integral absorption of PCR products (IA) was measured by Imagemaster VDS Image Analysis Software normalizing the data with GADPH internal control amplicons. The ratio of NP (IA) over GAPDH (IA) is represented as N(IA). The ratio of NPC (IA) over GAPDH (IA) is represented as T(IA). NAP-1 gene expression was considered low in NPC

Table 3: Expression difference between NPC and NP biopsies by differential RT-PCR in 40 cases

case number	N(IA)	T(IA)	T(IA) / N(IA)	case number	N(IA)	T(IA)	T(IA) / N(IA)
1	2.77	0.06	0.02	2	0.68	0.00	0.00
3	3.33	0.40	0.12	4	0.13	0.08	0.61
5	4.03	2.30	0.57	6	2.86	0.00	0.00
7	0.47	0.00	0.00	8	0.01	0.98	98.00
9	1.43	1.72	1.20	10	0.88	1.84	2.09
11	0.81	0.56	0.70	12	0.89	0.30	0.34
13	0.93	0.98	1.03	14	0.01	0.70	70.00
15	1.06	0.16	0.15	16	0.71	0.46	0.65
17	0.08	0.26	3.25	18	2.48	0.01	0.00
19	0.16	0.30	1.88	20	1.60	0.48	0.30
21	1.16	0.46	0.40	22	0.53	0.61	1.15
23	0.50	0.32	0.64	24	1.88	0.68	0.36
25	0.83	0.82	0.99	26	0.60	0.00	0.00
27	5.34	5.50	1.03	28	8.01	0.45	0.06
29	0.86	0.73	0.85	30	1.03	3.09	3.00
31	1.07	0.97	0.91	32	0.81	1.20	1.48
33	1.36	0.18	0.13	34	0.12	0.27	2.25
35	0.25	0.00	0.00	36	0.06	0.00	0.00
37	0.27	0.17	0.63	38	0.01	0.00	0.00
39	0.68	0.47	0.69	40	0.44	0.75	1.70

IA : Integral absorption of PCR products; N(IA): IA(N)/ IA (GAPDH); T(IA) :IA(T)/ IA (GAPDH); T(IA) / N(IA) < 0.5: expression decreased; T(IA) / N(IA) > 2: expression increased; 0.5< T(IA) / N(IA) < 2: no expression difference

if T(IA) over N(IA) was less than 0.5. Conversely, increased expression was considered a T(IA) / N(IA) ratio above 2. As a consequence no significant differences in expression between NPC and NP were included between T(IA) / N(IA) ration bigger than 0.5 and lower than 2.

Human Genome Draft Searching Method

The NAP-1 gene cDNA was identified from NP biopsies combining information derived through the Human Genome Draft Searching Method combined with nested PCR [3]. As a large-scale sequencing fragment could be identified in Human Genome Draft corresponding to the EST contig, we searched for stop codons in frame with the contig starting codon in the sequencing fragment upstream of the contig 5' end. Two forward primers were designed flanking the stop codon (pFRW-2, GATTTTGGTATTTGGTAGTTTC; pFRW-3, CTCCATTCCATTATACCTTTGAG) and two reverse primers were designed flanking the contig start codon (pRVS-3, GAGAGACTGGGAAACCAACAG; pRVS-2, GGAATTGGTGGAAGTGGGCGA). The fragment was amplified by PCR from cDNA of NP biopsies. Primers pFRW-2/ pRVS-3, pFRW-2/ pRVS-2, pFRW-3/ pRVS-3, and pFRW-3/ pRVS-2 were used for PCR amplifications. Amplifications were carried out in a Biometra thermal cycler according to the following program: 95°C for 3 minutes, 30 cycles of 94°C for 40 seconds,60°C for 30 seconds, 72°C for 50 seconds and a final extension at 72°C for 5 minutes. The resulting PCR products were run on agarose gels and stained with ethidium bromide. The theoretical sizes of PCR products predicted according to Human Genome Draft Searching Method were 176 bp (pFRW-3/ pRVS-3), 282 bp (pFRW-3/pRVS-2), 296 bp (pFRW-2/ pRVS-3) and 402 bp (pFRW-2/ pRVS-2) respectively. Gene identity was confirmed by concordance of predicted and actual amplicon size.

Northern Blot and Western blotting analysis

The probe for Northern Blot was prepared by PCR amplification of the pUCm-T/ NAP-1 plasmid and labeled using α-^{32}P-dCTP using the Prime-a-Gene Labeling System (Promega Corporation, Madison, WI, USA). The radioactivity of the probe is bigger than 1×10^8cpm/ μg. The probe was purified by Sephadex G-50 column and hybridized to human tissues according to a standard Northern blot procedure (Cat.7760-1 and Cat.7767-1, BD Biosciences Clontech, Palo Alto, CA, USA) using ExpressHyb™ Hybridization Solution (Cat.636831, BD Biosciences Clontech, Palo Alto, CA, USA).

Rabbit Anti-NAP-1 polyclonal antibody was prepared by Wuhan Boster Biological Technology Ltd., Wuhan, Hubei, China based on the N-terminus peptide sequence of the NAP-1 protein: GFPVSQDQEREKRSI.

The Phototope®-HRP Western Blot Detection Kit (New England Biolabs Inc., Beverly, MA, USA) was adopted for Immunoblotting analysis in FDC lines culture superna-

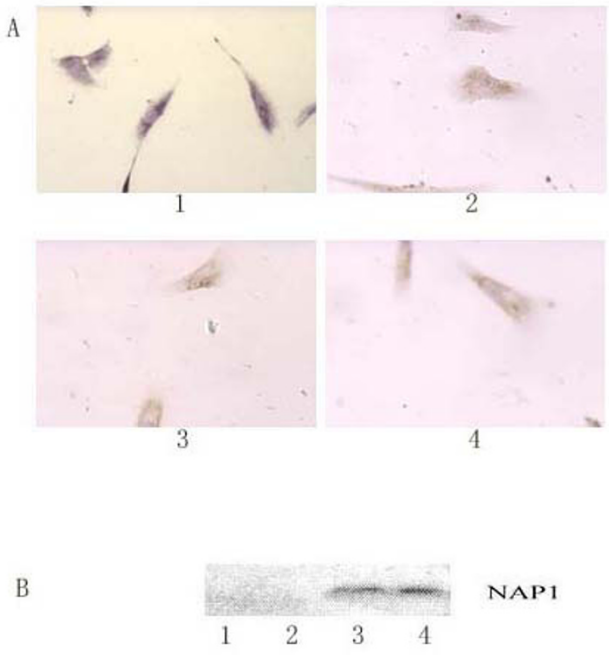

Figure 3
NAP-1 protein is a secreted protein secreted by FDC. A: NAP-1 gene is expressed in S100$^+$ CD35$^+$ FDC cell lines 1. In situ hybridization by Dig labelled NAP-1 cDNA probe in FDC lines (×300); 2. Immunohistochemistry by Mouse Anti-S100 Monoclonal antibody in FDC lines (×300); 3. Immunohistochemistry by Mouse Anti-CD35 Monoclonal antibody in FDC lines (×300); 4. Immunohistochemistry by Rabbit Anti-NAP-1 polyclonal antibody in FDC lines (×300) **B: Western blotting analysis of FDC culture supernatants using rabbit anti-NAP-1 polyclonal antibody** 1. The negative antibody control: the primary antibody replaced with block solution; 2. The negative supernatants control: the FDC lines culture supernatants replaced with fresh media; 3. Western blotting analysis of FDC supernatants cultured for 1 day; 4. Western blotting analysis of FDC supernatants cultured for 2 days

tants. Supernatants were collected after one and two days of FDC culture. Supernatants were concentrated by vacuum evaporation and collected according to a commercial Western Blot Detection Kit protocol. Protein samples and molecular weight standards were separated by polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose (NC) membrane by electroblotting and blocked to saturate nonspecific binding sites on the membrane. The membrane was incubated with the primary rabbit anti-NAP-1 polyclonal antibody followed by incubation with HRP-linked anti-rabbit IgG. LumiGLO™ reagent was then added and the emission was captured on X-ray films. NC membranes with the primary antibody replaced with block solution were used as negative controls. Fresh culture media was used as negative control for the supernatants.

In situ hybridization and Immunohistochemistry

NP and NPC biopsies were immediately fixed following surgical excision to prevent degradation of messenger RNA. The tissues were incubated at 4°C for 2–4 hours with freshly made, filtered fixative (DEPC-treated PBS containing 4% paraformaldehyde; pH 7.5) and stored in a freezing compound at -80°C. Samples were then warmed to -20°Cm cut in 10 μm sections in a cryostat and placed on pretreated glass slides for analysis. The slides were Dried in an oven at 40°C overnight and used immediately or stored in a box at -80°C. Before processing, the stored slides were warmed at room temperature and dries in an oven at 40°C for a minimum of 2 hours.

FDC cells were cultured at 37°C in a 5% CO_2 atmosphere on poly-L-lysine coated microscopic slides in Dulbecco's minimal essential medium without phenol red. Cells were washed with PBS at 37°C and fixed at room temperature for 30 minutes in a 4% (w/v) formaldehyde, 5% (v/v) acetic acid, and 0.9% (w/v) NaCl solution. Fixed cells were washed with PBS at room temperature and stored in 70% ethanol at 4°C.

In situ hybridization was performed according to the Dig DNA Labelling and Detection Kit's protocol (Boehringer Mannheim Gmbh Biochemica, Mannheim, Allemagne, Germany). Briefly, a NAP-1 specific cDNA probe was prepared by PCR DIG Probe Synthesis. The prepared tissue sections and FDC cells on slides were treated with proteinase K (Sigma-Aldrich Corporation., Saint Louis, MO), washed, pre-hybridized, and then hybridized overnight at 50°C with 20 μl hybridization buffer containing 30 ng DIG-Labeled probe. After hybridization, slides were rinsed, and washed twice with 2 × SSC, 1 × SSC and 0.1 × SSC at 50°C for 15 minutes each. After washing, the slides were stained with Fast Red if need. Hybridization buffer containing no DIG-Labeled probe was used as negative control.

The primary CD35, CD3, CD20, CD57, CD68, S-100 monoclonal antibodies (Zymed Laboratories, Inc., South San Francisco, CA) and the rabbit Anti-NAP-1 polyclonal antibody were used for Immunohistochemistry. The "two steps" system (Antibody Diagnostica Inc., Stamford, CT) was used. The slides were incubated with 3% H_2O_2 at room temperature for 10 minutes to inactivate internal enzymes and then washed with PBS (pH7.6). Normal sheep serum was added in droplets. Primary antibodies were diluted with PBS (1 : 50) and added to the slides,

which were then placed in the refrigerator (4°C) overnight. Then, the HRP-linked anti-rabbit IgG or HRP-linked anti-mouse IgG (Antibody Diagnostica Inc.) were added. Slides were incubated at 37°C for 40 minutes and then washed 3 times after each step for 5 minutes. Then the slides were stained with DAB enzyme for 5–30 minutes, dehydrated, made transparent with dimethylbenzene and sealed with neutral gum. They were then visualized under a microscope. Slide with the primary antibody replaced with PBS were used as the negative control.

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